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Expression in *Saccharomyces cerevisiae* of a gene associated with cytoplasmic male sterility from maize: Respiratory dysfunction and uncoupling of yeast mitochondria

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Summary. We asked whether the mitochondrial T-*urf13* gene, associated with the male sterility phenotype of T cytoplasm in maize, can be expressed in *Saccharomyces cerevisiae* and whether this expression can mimic the effects observed in maize. We introduced the universal code equivalent of the T-*urf13* gene into the *S. cerevisiae* nucleus by transformation and directed its translation product into mitochondria by means of a fusion with the targeting presequence from *Neurospora crassa* ATPase subunit 9. We show that expression of the universal code equivalent of the T-*urf13* gene in the yeast nucleus does indeed mimic its effects in maize: respiratory growth of yeast is inhibited, respiration-deficient cytoplasmic mutants accumulate and NADH oxidation of isolated mitochondria is uncoupled. All these effects are observed only if the mitochondrial targeting peptide and methomyl or HmT toxin are present.

Key words: *Cochliobolus heterostrophus* race T disease – Maize cytoplasmic male sterility – Mitochondria – *Saccharomyces cerevisiae*

Introduction

Cytoplasmic male sterility (cms) in plants is a phenomenon of considerable interest both for fundamental and applied research (for review see Lonsdale 1987; Pring et al. 1988). Of the three types of sterility (C, S and T) described in maize (*Zea mays* L.), which are distinguished by their nuclear restorer genes (Beckett 1971), the one displayed by the Texas (T) cytoplasm has been studied most extensively. T-cms is associated with and probably due to the presence in maize mitochondrial DNA (mt DNA) of a 345 bp chimeric gene, T-*urf13*, composed primarily of sequences derived from 26S rDNA coding and 3′ regions (Dewey et al. 1986). This gene is also associated with maternal inheritance of sensitivity to disease toxin (*Cochliobolus heterostrophus* race T toxin, HmT toxin) and to the carbamate insecticide methomyl (see review by Pring and Lonsdale 1989). T-*urf13* is deleted (Fauron et al. 1987; Rottmann et al. 1987; Wise et al. 1987a) or truncated through a frameshift mutation (Wise et al. 1987a) in mutants to male fertility and disease resistance derived in maize tissue culture. A 13 kDa polypeptide is the mitochondrial product of the gene (Forde et al. 1978; Dewey et al. 1987; Wise et al. 1987b). The role of the 13 kDa polypeptide in cms is not completely understood but it is believed that T-cms results from an impairment of mitochondrial activity which can be demonstrated in the presence of the toxin or methomyl. It has been found that maize mitochondria isolated from T cytoplasm display, in the presence of HmT toxin or methomyl, increased membrane permeability to Ca^{++} (Holden and Sze 1984), leakage of NAD^{+} and CoA (Matthews et al. 1979; Bervillé et al. 1984), irreversible swelling (Klein and Koeppe 1985), loss of the transmembrane potential (Bervillé et al. 1984) and various changes in the rate of substrate oxidation, in particular a dramatic uncoupling of NADH oxidation (Miller and Koeppe 1971; Gengenbach et al. 1973; Flavell 1975; Yoder et al. 1977; Gregory et al. 1978; Koeppe et al. 1978; Bervillé et al. 1984; Klein and Koeppe 1985). This is consistent with the demonstration that the T-*urf13* gene, when expressed in *Escherichia coli*, confers HmT toxin and methomyl sensitivity on the bacterium (Dewey et al. 1988): in particular, swelling and inhibition of whole cell respiration are observed in the presence of toxin or methomyl. Recently, site-directed mutagenesis of the T-*urf13* gene has localized critical positions in the protein necessary for its deleterious effects in *E. coli* (Braun et al. 1989).

We asked whether the T-*urf13* gene of maize mtDNA can be expressed in the yeast *Saccharomyces cerevisiae* and whether the effects of this expression mimic those observed in maize. To test this we introduced the T-*urf13* gene from the maize mitochondrial genome into the *S. cerevisiae* nucleus by transformation and directed its

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translation product into yeast mitochondria. Several steps were required to achieve these goals: (i) the mitochondrial genetic code of higher plants differs from that of the *S. cerevisiae* nuclear genome where the CGG codon (edited in maize mitochondrial RNA to UGG, i.e. tryptophan, Covello and Gray 1989; Gualberto et al. 1989) specifies arginine; we thus changed it to TGG so that the cognate amino acid, tryptophan, would be present in the protein. (ii) Several proteins encoded in nuclear DNA are imported into mitochondria by virtue of an N-terminal, targeting peptide (Schatz 1987; Attardi and Schatz 1988); we added a sequence coding for such a targeting peptide to the T-*urf13* gene. (iii) Specific signals for *S. cerevisiae* transcription were added to flank the T-*urf13* gene in order to obtain high-level expression in yeast.

We show that expression of the universal code equivalent of the T-urf13 gene in the yeast nucleus does indeed mimic its effects in maize mitochondria: respiratory growth of yeast is inhibited, respiration-deficient cytoplasmic mutants (*rho*$^-$ "petites") accumulate and NADH oxidation in isolated mitochondria is uncoupled. All these effects are observed only if the mitochondria-targeting N-terminal peptide *and* methomyl or HmT toxin are present.

Materials and methods

Strains and plasmids. The *S. cerevisiae* strain CW04 used in this work has the nuclear genome of W303-1B α *ade2-1, ura3, his3-11, 15, trp1-1, leu2-3, 112, can*^{r} (Banroques et al. 1986) and carries the wild-type mitochondrial genome rho$^+$ mit$^+$ of strain 777-3A (Dujardin et al. 1980). Recombinant plasmids were selected in *E. coli* strain TG1, K12, Δ (*lac−pro*), *supE, thi, hsdD5*/F′, *traD36, proA*$^+$*B*$^+$, *lacI*^{q}, *lacZ*, Δ M15. Plasmids used were pT-H18 (Wise et al. 1987a), T-urf13/M13 (this work), pUCurf13-TW/bh (this work), pEMBLYe30/2 and its derivative pEMBLYe30/2-21-0 (Banroques et al. 1986).

Oligonucleotide site-directed mutagenesis. Mutagenesis was carried out using the oligonucleotide site-directed in vitro system (Amersham) based on the method developed by Taylor et al. (1985a, b). The mutagenic oligonucleotides were annealed to the single-stranded template and extended by Klenow polymerase in the presence of T4 ligase to generate a mutant heteroduplex. Selective removal of the non-mutant strand was made possible by incorporation of a thionucleotide into the mutant strand during in vitro synthesis. Digestion by *Nci*I, a restriction enzyme that cannot cleave phosphorothioated DNA, generated single-strand nicks in the non-phosphorothioated strand. Such nicks present sites for Exonuclease III which was used to digest the non-mutant strand. The mutant strand was used as a template to reconstruct the double-stranded closed circular molecule, thus creating a homoduplex mutant molecule.

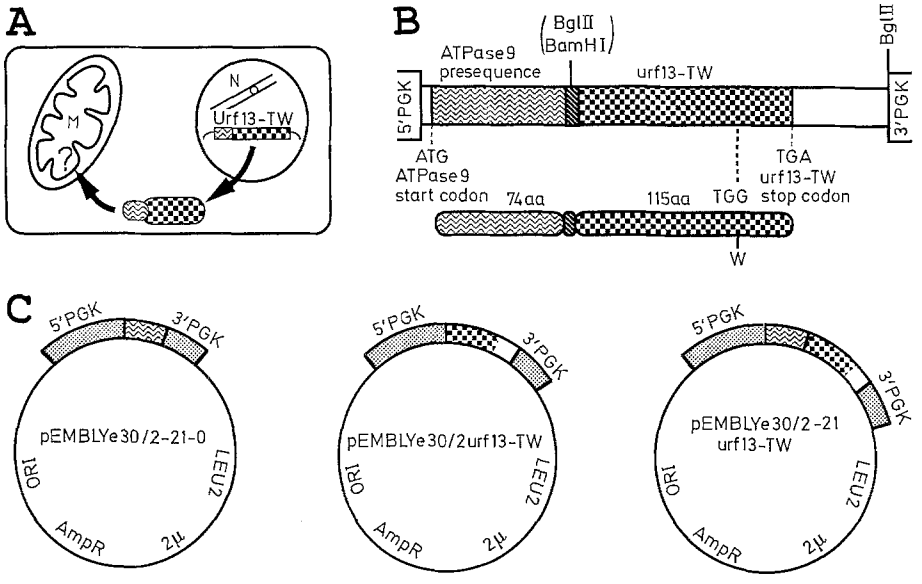
Oligonucleotides were synthesized using an automatic DNA synthesizer (Milligen model 7500) and were purified by polyacrylamide gel electrophoresis.

Construction of the chimeric gene. The maize T cytoplasm mtDNA clone from pT-H18 (Wise et al. 1987a) was used in the construction of the TGG-87 *urf13-TW* gene. The entire 2 kb *Hin*dIII fragment carrying the T-*urf13* open reading frame, was cloned into the M13mp18 polylinker *Hin*dIII site to give T-urf13/M13. By in vitro oligonucleotide site-directed mutagenesis, the CGG codon at position 87 was changed to a TGG codon by using a 21-mer oligonucleotide with the following sequence: 5′ CCTTCATTCCATTGTATAGGT 3′, thus giving the *urf13-TW* gene. The modification was sequenced (data not shown) using the method of Sanger et al. (1977).

Next a ligation site for the insertion of *urf13-TW* into yeast nuclear vectors was created. By in vitro oligonucleotide site-directed mutagenesis an *Nde*I site was created upstream from the *urf13-TW* start codon by using a 24-mer oligonucleotide with the following sequence: 5′ GTAGTGATCATATGACGAAAAAGT 3′. After *Nde*I digestion of the modified double-stranded urf13-TW/M13 and Klenow polymerase treatment to generate blunt ends, the 1.3 kb fragment carrying the *urf13-TW* coding sequence was extracted from a 0.8% agarose gel and purified. After addition of an appropriate *Bam*HI linker to maintain the phase, digestion of this fragment with *Bam*HI and *Hin*dIII generated a 0.8 kb fragment which was cloned into pUC19 to give pUCurf13-TW/bh.

Finally, *urf13-TW* was fused with the mitochondrial targeting presequence. *Urf13-TW* was cloned as a 0.5 kb *Bam*HI−*Bgl*II fragment from pUCurf13-TW/bh into the unique BglII site of the yeast nuclear vectors pEMBLYe30/2-21-0 and, as a control, pEMBLYe30/2 (Banroques et al. 1986). These two vectors carried the 5′ and 3′ transcription control regions (promoter and terminator respectively) of the yeast phosphoglycerate kinase (PGK) gene, which is expressed at high levels (Mellor et al. 1983). In addition pEMBLYe30/2-21-0 (control plasmid 1, see Fig. 1) carried, immediately downstream of 5′ PGK, the sequence (Viebrock et al. 1982) encoding the 74 N-terminal amino acids of *Neurospora crassa* ATPase9, which comprise the mitochondrial targeting presequence. The unique *Bgl*II site is located downstream from this sequence. The fusion of the *urf13-TW* gene in phase with the ATPase9 presequence, giving pEMBLYe30/2-21urf13-TW (active plasmid, see Fig. 1), created five additional codons resulting from the construction of the vector (Banroques et al. 1986) and the manipulation of the *urf13-TW* 5′ end described above. The insertion of *urf13-TW* into the unique *Bgl*II site in pEMBLYe30/2 (located downstream from the PGK promoter), generated pEMBLYe30/2urf13-TW (control plasmid 2). All plasmids cited carried the information required for autonomous replication and selection in both *E. coli* and *S. cerevisiae* (*ori*, *Amp*^{r} and *2 μ*, *LEU2* respectively).

Media. Synthetic fermentable medium was composed of 0.67% Difco yeast nitrogen base, 2% glucose. Synthetic respirable medium contained 0.67% Difco yeast nitrogen base, 1% glycerol, 1% ethanol and 0.05% glucose



CONTROL PLASMID 1 CONTROL PLASMID 2 ACTIVE PLASMID

Fig. 1A–C. The design of the experiment to study the effects of expression in yeast of the maize *T-urf13* gene, associated with Texas cytoplasmic male sterility (T-cms). **A** The yeast nucleus was transformed by a replicative plasmid containing a chimeric gene construct (**B**) composed essentially of the mitochondria-targeting N-terminal peptide (from the nuclear gene encoding ATPase subunit 9 of *Neurospora crassa*) linked to the gene associated with T-cms (i.e. *T-urf13*) from the maize mitochondrial genome. The *T-urf13* gene had previously been adapted to the universal genetic code by oligonucleotide site-directed mutagenesis changing the CGG-87 codon (arginine in yeast) to TGG-87 codon (tryptophan in yeast) giving *urf13-TW*. The expression of this chimeric gene would lead to the synthesis of a chimeric protein which could, by virtue of its targeting presequence, deliver the 13 kDa protein coded by *urf13-TW* to the yeast mitochondria. **B** Details of the chimeric construction. 5'PGK and 3'PGK were yeast transcriptional signals (promoter and terminator respectively) giving high-level constitutive expression, derived from the phosphoglycerate kinase gene (Mellor et al. 1983). 5'PGK was linked to a fragment containing 222 bp of the *N. crassa* ATPase9 presequence (Viebrock et al. 1982) as in Banroques et al. (1986) and further linked in phase to the *urf3-TW* sequence derived from *T-urf13* (Dewey et al. 1986). The gene fusion created five additional codons between the GCC-74 codon of the ATPase9 presequence and the ATG-1 start codon of *urf13-TW*, essentially because of the addition of a *Bam*HI linker (see Materials and methods). The resulting chimeric protein was composed of the first 74 amino acids of the *N. crassa* ATPase9 presequence (positions 1 to 74) followed by 5 new amino acids (positions 75 to 79) and by the entire 115 amino acids of *urf13-TW* (positions 80 to 194) with a tryptophan-166 (position 87 in T-*urf13*) as summarized below: $M^{1}AST \ldots AQA^{74}M^{75}GRSG\text{-}^{79}M^{80}ITT \ldots W^{166} \ldots QVP^{194}Ochre$. **C** Organisation of the active plasmid and the two control plasmids. The active plasmid contained the complete chimeric gene construct described in **B**. The two control plasmids contained either the ATPase9 presequence alone (control plasmid 1) or the *urf13-TW* gene alone (control plasmid 2). All the plasmids were identical with respect to the replication and selection functions required in *Saccharomyces cerevisiae* and *Escherichia coli* and differed only in this insert constructs. *Wavy lines* ATPase9 presequence; *black areas with white hatching* additional domains; *chequered areas urf13-TW*

to initiate growth. All media were supplemented with appropriate auxotrophic requirements when needed.

Yeast transformation. After transformation using the lithium procedure, (Ito et al. 1983), the transformed cells were selected for leucine prototrophy on synthetic fermentable medium.

Tetrazolium yeast staining. Yeast were stained using 2,3,5-triphenyl tetrazolium chloride as described by Ogur et al. (1957).

Measurement of growth rate. Yeast growth was followed by using a Klett-Summerson photoelectric colorimeter. Klett flasks containing 10 ml of medium were inoculated with the equivalent of 10–15 Klett units and incubated at 28° C with agitation. Precultures were made in synthetic fermentable medium and were washed twice in 0.4% NaCl solution before inoculation.

Extraction and purification of yeast mitochondria. Extraction was performed essentially according to Villalobo et al. (1981) with the modifications described below. Yeast cells were grown at 28° C with agitation to the end of log phase in 4 l of synthetic respirable medium containing 0.67% Difco yeast nitrogen base, 1% glycerol, 2% ethanol, 0.05% glucose, 0.2% KH_2PO_4, 0.2% $(NH_4)_2SO_4$ with all auxotrophic requirements except leucine. Cells were harvested and washed with distilled water. The pellet was resuspended in 100 ml of 10 mM Tris-HCl pH 8.5, 100 mM β-mercaptoethanol and incubated for 30 min at 33° C. Cells were harvested and washed twice with distilled water and once with digestion buffer (1.3 M sorbitol, 10 mM imidazole-HCl pH 6.4, 0.5 mM EDTA, 0.2% BSA). The pellet was resuspended in 50 ml of digestion buffer. Then 300 μl of glusulase (Serva) and 0.5 mg/g of cells (wet weight) of zymolyase 100000 (Seikagaku kogyo) were added. After 30 min of incubation at 33° C, spheroplasts were harvested and

washed twice with digestion buffer. Spheroplasts were lysed by resuspension in 20 ml of 0.3 M sorbitol, 10 mM imidazole-HCl pH 6.4, 0.2% BSA. Then 20 ml of 1 M sorbitol, 10 mM imidazole-HCl pH 6.4, 0.2% BSA was added before the lysate was centrifuged for 5 min at 2000 *g* at 4° C. The supernatant was centrifuged for 20 min at 18000 *g* at 4° C. The mitochondrial pellet was resuspended gently in 30 ml of mitochondrial buffer (0.6 M mannitol, 10 mM imidazole-HCl pH 6.4, 0.2% BSA) and centrifuged for 5 min at 2000 *g* at 4° C to remove the remaining cellular debris. The supernatant was centrifuged once more for 20 min at 18000 *g* at 4° C. The final mitochondrial pellet was resuspended gently in a minimal volume of mitochondrial buffer. The mitochondrial suspension was aliquoted, rapidly frozen in liquid nitrogen and stored at −70° C.

Measurement of oxygen uptake. Yeast cells were grown for 48 h at 28° C in the synthetic leucine-free respirable medium. Oxygen uptake by intact yeast cells was measured using a Clark electrode (Gilson Oxygraph Model IC OXY) in a closed compartment containing 2 ml of measurement medium (1% Difco yeast extract, 2% Difco bactopeptone, 4% glycerol, adjusted to pH 5.8) and maintained at 28° C. Measurements were made with approximately 5×10^7 to 7.5×10^7 cells/ml, maintained in suspension with a magnetic stirrer. The final concentration of methomyl was 6 mM.

Oxygen uptake by yeast mitochondria was measured in the same way but using 2 ml of the following buffer: 0.6 M mannitol, 10 mM imidazole-HCl pH 6.4, 10 mM KH_2PO_4, 7.5 mM $MgCl_2$, 0.2% BSA. Measurements were made with 0.4 mg/ml of yeast mitochondrial protein and the suspension was kept homogeneous with a magnetic stirrer. The final concentrations of substrates were 2 mM NADH, 20 mM succinate and 30 mM malate/citrate. The final concentrations of drugs were 20 µM carbonylcyanide *m*-chlorophenylhydrazone (CCCP), 10 µM antimycin A and 6 mM methomyl.

Disc tests of toxin sensitivity. Yeast transformants were grown overnight at 28° C in the synthetic fermentable medium without leucine, washed in 0.4% NaCl solution and resuspended in fresh synthetic fermentable medium. The equivalent of 1 ml of culture was added to a molten agar solution (precooled at 45° C) and poured on a synthetic fermentable medium plate without leucine. After addition of a sterile paper disc, methomyl (dissolved in water, sterilized by filtration) or HmT toxin was added to each disc. Plates were incubated 24 h at 28° C and stained with tetrazolium for 2 h in the dark (Ogur et al. 1957). Respiration-competent cells reduced the dye to a red-colored formazan while their absence led to a white halo surrounding the disc.

Results

Construction of the chimeric gene and controls

Urf13-TW, the universal genetic code equivalent of the T-*urf13* gene associated with T-cms is fused in phase to the sequence encoding the mitochondria-targeting N-terminal peptide from *N. crassa* ATPase subunit 9 (Fig. 1 B and Materials and methods). The ATPase9 presequence has already been successfully used (Banroques et al. 1986) and contains the signals required to target foreign protein into the mitochondrial inner membrane (Douglas et al. 1986) which is the putative site of action of the T-urf13 protein in T cytoplasm maize mitochondria (Dewey et al. 1987; Hawkesford and Leaver 1987). This complete chimeric gene construct is carried on pEMBLYe30/2-21urf13-TW (the active plasmid; Fig. 1 C). Its transcriptional control is ensured by the 5′ and 3′ regions (promoter and terminator respectively) of the highly expressed yeast PGK gene (Mellor et al. 1983) and its translation product would, with the help of the presequence, be important into yeast mitochondria (Fig. 1 A). As control constructs, the sequences encoding the ATPase 9 N-terminal peptide and the *urf13-TW* gene are carried separately by control plasmids 1 and 2 respectively with the same transcriptional signals (Fig. 1 C) to test the effects of the presequence alone and the effects of the *urf13-TW* gene when its translation product is not targeted into yeast mitochondria.

Inhibition of respiratory growth and induction of petites

Yeast was transformed (Ito et al. 1983) to leucine prototrophy either by the active plasmid carrying the complete chimeric gene construct or control plasmids 1 and 2 carrying the ATPase 9 N-terminal peptide sequence and the *urf13-TW* gene, respectively (see Fig. 1 C). Sensitivity to HmT toxin and methomyl of the three types of transformants was investigated in disc test experiments (Fig. 2). Expression of the *urf13-TW* gene in yeast confers sensitivity to HmT toxin and methomyl only when the *urf13-TW* gene is fused to the *N. crassa* ATPase 9 N-terminal peptide sequence. Sensitivity is indicated by development of a white halo surrounding the disc when plates are stained with tetrazolium (Ogur et al. 1957) (Fig. 2 A). The halo is due to the absence of cell multiplication and/or to the absence of cell respiration, since the pigment is formed only by respiration-competent cells. Control transformants are not significantly affected by the two toxic agents as shown by the fact that all cells stain with tetrazolium; the whole plate including the disc and its surrounding area takes on a red color (see Fig. 2 B, C).

When the growth of transformants carrying the active plasmid is followed in leucine-free fermentable medium, a significant decrease in the growth rate is observed in the presence of methomyl (the doubling time goes up to 13 h in the presence of 3 mM methomyl compared with 2.5 h in its absence, data not shown) while the growth of control transformants is not affected. The proportion of *rho*$^-$ (petite) mutants increases (Table 1) when the *urf13-TW* gene is expressed as a fusion product with the ATPase 9 N-terminal peptide sequence while it remains low and constant in the control transformant. This increase is greater when the methomyl concentration is increased. This is supported by the study of the

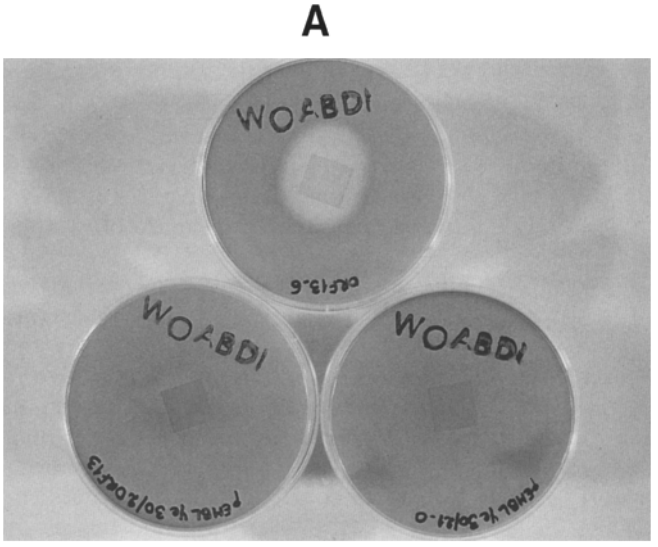


Fig. 2A–C. Disc tests of sensitivity to methomyl and HmT toxin of yeast carrying active and control plasmids. The yeast strain CW04 was transformed with the active plasmid carrying *urf13-TW* fused to the ATPase9 presequence (**A**), with control plasmid 1 carrying the ATPase9 presequence alone (**B**) or with control plasmid 2 carrying *urf13-TW* alone (**C**). Transformants grown overnight at 28° C in synthetic fermentable medium were tested for sensitivity to 4.8 mg methomyl in discs as described in Materials and methods. Sensitivity is indicated by a white halo representing lack of respiration competence (**A**). Respiration-competent cells (**B**, **C**) reduce the tetrazolium dye used to red-colored formazan. Experiments using 0.5 to 8 mg methomyl or 0.1 or 1 μg HmT toxin gave the same results

Table 1. The expression of the complete chimeric gene induces cytoplasmic "petite" mutants in yeast

Methomyl (mM)	Percentage respiration-deficient cytoplasmic mutants	
	+urf13-TW	Control
0	1.5	4.0
3	21.5	3.8
6	38.2	2.0

The *rho*$^+$ yeast strain CW04 was transformed with the active plasmid carrying *urf13-TW* fused to the ATPase9 presequence or by control plasmid 1 carrying the ATPase9 presequence alone (see Fig. 1). The two types of transformants were grown at 28° C for 2 days in synthetic fermentable medium without leucine and with various methomyl concentrations, plated on complete fermentable medium and replicated on complete respirable medium. The proportion of respiration-deficient mutants (small size colonies on fermentable medium and no growth on respirable medium) increased with methomyl concentration when the cytoplasmic male sterility gene was present, while it remained low and constant in its absence. The percentage of petites was determined from counts of more than 200 colonies

respiratory growth of the two types of transformants. Ethanol and glycerol are used as respirable substrates at concentrations of 1% each (0.05% glucose is furnished to initiate growth and its utilization can be subtracted by following the growth in control medium where it is the sole carbon and energy source, see Fig. 3). Complete inability to use respirable substrates is observed when yeast cells transformed with the active plasmid are grown in the presence of 6 mM methomyl, partial inability is found in 3 mM methomyl and only a very small decrease in respiration is observed in its absence. However, neither inhibition nor stimulation of the oxygen uptake of intact cells was observed after methomyl addition to transformants carrying the active plasmid or control plasmid 1 (Fig. 4).

Another fusion of the *urf13-TW* gene with a different mitochondria-targeting presequence was constructed (data not shown), using the yeast nuclear vector pEMBLYe30/2-23-0 (Banroques et al. 1987). This fusion is identical to active plasmid except for the mitochondria-targeting N-terminal peptide which is encoded by a 36 bp sequence corresponding to the first 12 N-terminal amino acids of the 70 kDa yeast mitochondrial outer membrane protein. This 12 amino acid peptide carries the signals required to target foreign protein into the yeast mitochondrial matrix (Schatz 1987) and has previously been used successfully (Hurt et al. 1985; Banroques et al. 1987). No sensitivity is observed when yeast is transformed with this chimeric gene construct (data not shown). Consequently, expression of the maize chimeric gene and targeting of its translation product into the yeast mitochondrial inner membrane confers the sensitivity to the HmT toxin and methomyl which appears as mitochondrial dysfunction, i.e. inhibition of respiratory growth and accumulation of respiration-deficient cytoplasmic mutants.

Methomyl causes dysfunction of mitochondria isolated from yeast expressing the complete chimeric construct

Why is the respiratory growth of yeast expressing the complete *urf13-TW* construct inhibited in the presence of methomyl (Fig. 3) while oxygen uptake is not (Fig. 4)? To investigate this we first isolated mitochondria from yeast carrying either the active plasmid or the control plasmid and grown in the absence of methomyl (as shown in Fig. 3, there is practically no difference in the respiratory growth of cells carrying the active or the control plasmids when methomyl is absent). Then, we analyzed various mitochondrial functions of the isolated organelles in the presence or absence of methomyl. Isolated mitochondria are strongly coupled as shown by the fact that the classic uncoupler, CCCP, increases the oxygen uptake by some 540% for both types of mitochondria when NADH is the substrate (Fig. 5B). In general, in the absence of methomyl, we could not detect any significant difference between the mitochondria isolated from the two types of cells. However, in the presence of methomyl major differences appear. Under all conditions studied, methomyl addition to control mitochondria induces a small decrease (15%–20%) in their oxygen uptake. On the other hand, effects of methomyl addition to *urf13-TW* mitochondria vary, depending on

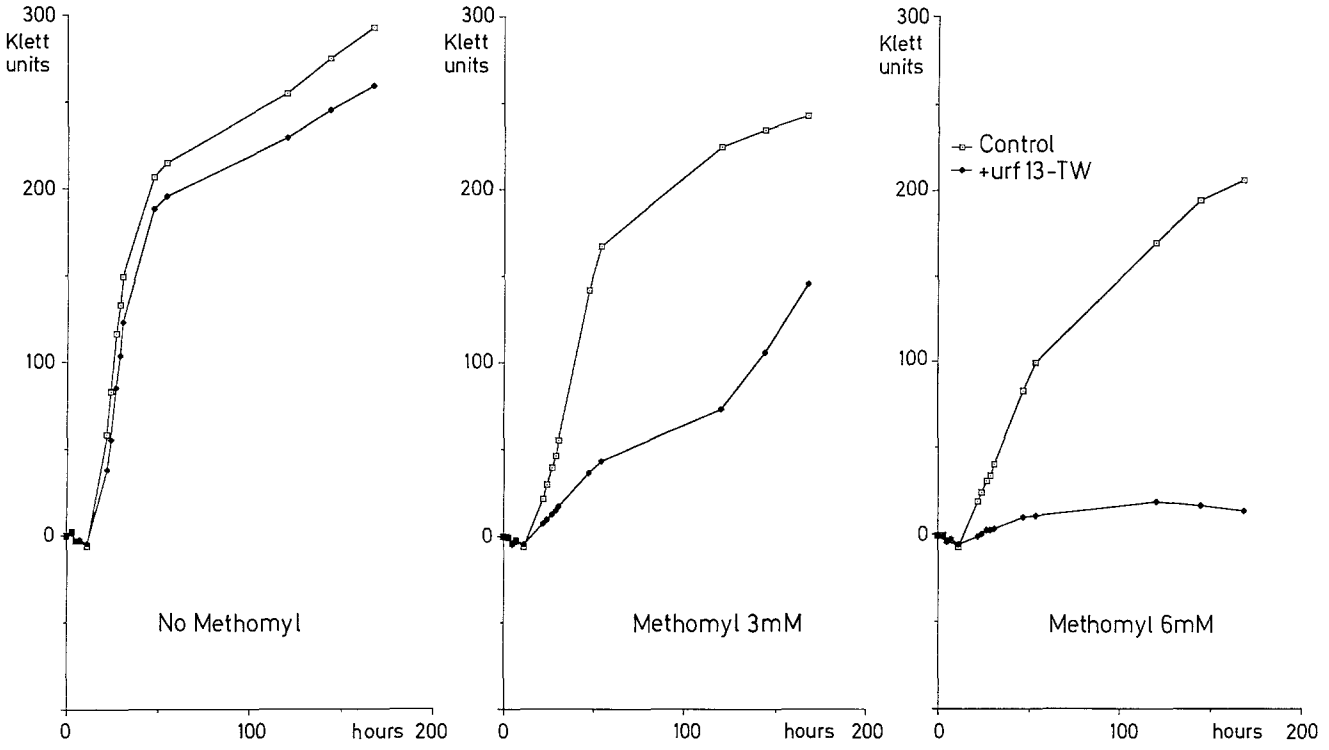


Fig. 3. Expression of the *urf13-TW* gene prevents respiratory growth of yeast in the presence of methomyl. The *rho*$^+$ yeast strain CW04 was transformed by the active plasmid carrying *urf13-TW* fused to the ATPase 9 presequence or by the control plasmid 1 carrying the ATPase 9 presequence alone. The growth of the two types of transformants was followed in synthetic respirable medium without leucine (containing 1% ethanol, 1% glycerol with 0.05% glucose) with various methomyl concentrations. As a control, growth was followed in the same synthetic medium containing 0.05% glucose alone. The growth in the control synthetic medium was subtracted from the growth in the synthetic respirable medium so that the graphs shown result from the utilisation of respirable substrates (ethanol and glycerol) only

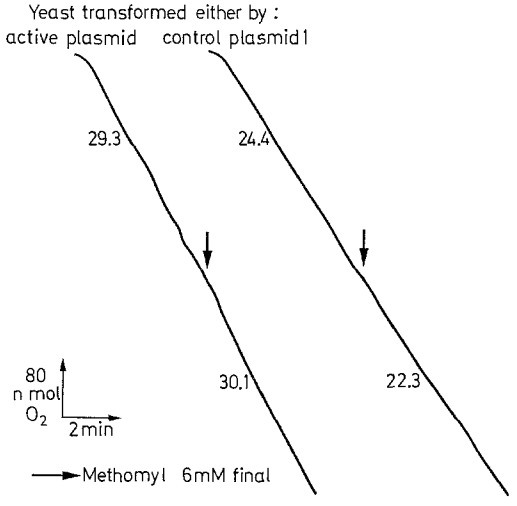


Fig. 4. Oxygen uptake of cells carrying the cytoplasmic male sterility associated gene is not modified by the addition of methomyl. The *rho*$^+$ yeast strain CW04 transformed either with the active plasmid or control plasmid 1 were grown (as in Fig. 3) in the absence of methomyl, harvested and resuspended in measurement medium (see Materials and methods) at a final concentration of 2.5×10^9 cells/ml. Oxygen uptake tracings were followed for at least 5 min. The addition of methomyl is indicated by *arrows*. Oxygen uptake (number under the traces) was expressed in nmol O_2/min per 5×10^7 cells

the substrate being oxidized. NADH oxidation of *urf13-TW* mitochondria (Fig. 5A and 5B left) drastically increases when methomyl is added. A 315% increase in oxygen uptake is observed, similar to the uncoupling effect of the addition of CCCP alone. This stimulation is sensitive to antimycin A (data not shown) since addition of antimycin A, following methomyl addition, completely inhibits the oxygen uptake. Methomyl acts in a different manner on *urf13-TW* mitochondria when succinate is the substrate. A decrease of 32% in oxygen uptake is observed instead of a strong stimulation as with NADH. This inhibition does not significantly differ from the effect observed on the control mitochondria. However, the uncoupling effect of CCCP addition following methomyl addition, is inhibited in *urf13-TW* mitochondria but not in control mitochondria (Fig. 5B right).

Discussion

We have introduced the universal code equivalent of the T-*urf13* gene from maize mitochondria into the *S. cerevisiae* nuclear genome and expressed it as a fusion protein with a mitochondria-targeting N-terminal peptide. The yeast cells become sensitive to the HmT toxin/methomyl

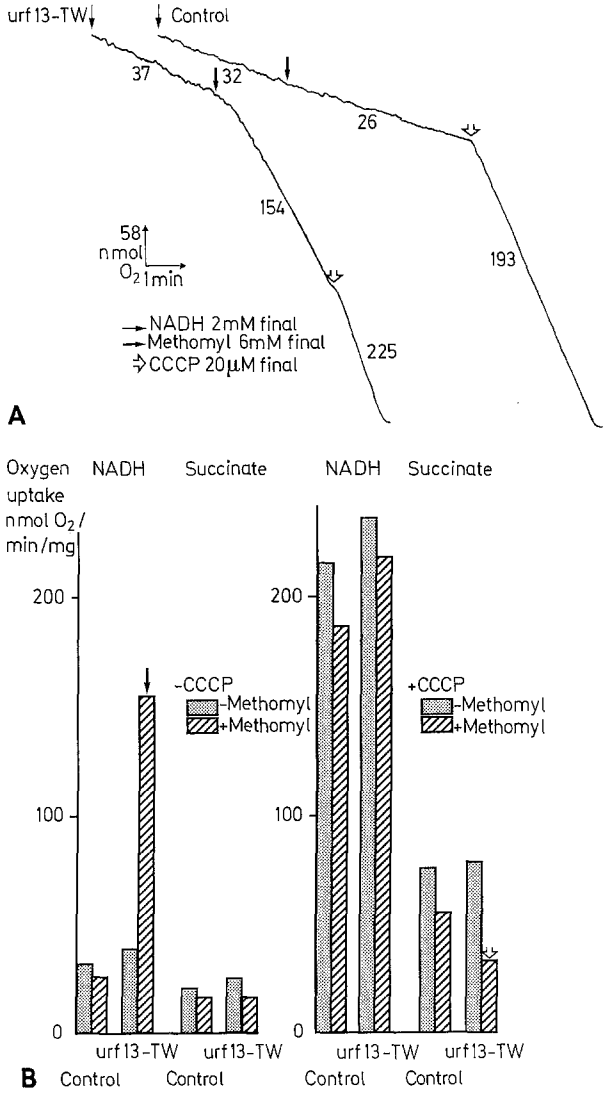


Fig. 5A and B. Effect of methomyl on NADH oxidation in yeast mitochondria isolated from cells carrying the cytoplasmic male sterility associated gene. **A** NADH oxidation traces. **B** Summary of effects on NADH and succinate oxidation (average results of 3 independent measurements). Yeast mitochondria were extracted and purified from the *rho*$^+$ yeast strain CW04 transformed either with the active plasmid (*urf13-TW*) or plasmid 1 (*control*; see Fig. 1) and grown in the absence of methomyl. Mitochondria (approximately 0.8 mg of protein) were incubated in measurement buffer for 4 min and the slope of oxygen uptake tracings was followed for at least 4 min. Sequential additions of NADH (or succinate), carbonylcyanide *m*-chlorophenylhydrazone (CCCP) are indicated by arrows (**A**). Oxygen uptake (numbers under the traces, **A**) is expressed in nmolO_2/min per mg mitochondrial protein. The *black arrow* in **B** indicates the increase in NADH oxidation after methomyl addition to *urf13-TW* mitochondria. The *white arrow* in **B** indicates the inhibition of the uncoupling effect of CCCP on succinate oxidation following methomyl addition in *urf13-TW* mitochondria. For further discussion, see text

in a manner that mimics the diagnostic phenotype of male sterile plants. Previous work has shown that the T-urf13 protein is intimately involved in the HmT toxin/methomyl sensitivity of a genetically engineered prokaryote (Dewey et al. 1988). Our work extends this conclusion to a eukaryote model, yeast, and shows that the target of the sensitivity is the mitochondrion itself, since the urf13-TW protein without an appropriate targeting N-terminal peptide has no effect at all. Furthermore, the urf13-TW protein fused to a different mitochondria-targeting N-terminal peptide, derived from the 70 kDa outer membrane protein is inactive. This indicates that the specific localization within the organelle is essential for the deleterious action of the maize protein. The first 12 N-terminal amino acids of the yeast mitochondrial outer membrane 70 kDa protein most probably target the passenger proteins into the mitochondrial matrix while the *N. crassa* ATPase 9 N-terminal peptide should target it into the inner membrane, as suggested by previous experiments (Hurt et al. 1985; Douglas et al. 1986; Banroques et al. 1986, 1987). Consequently, the urf13-TW protein would confer sensitivity on yeast mitochondria when inserted into the mitochondrial inner membrane but not when present in the mitochondrial matrix. This is consistent with the observations made on maize. Immunological evidence suggests that the T-urf13 protein is primarily associated with the mitochondrial membranes (Dewey et al. 1987) in particular with the fraction enriched in subunit I of cytochrome oxidase which is an integral part of the inner membrane (Hawkesford and Leaver 1987).

The presence of the complete construct (*urf13-TW* fused to the *N. crassa* ATPase 9 N-terminal peptide) has two major effects in yeast which are revealed by in vivo and in vitro studies. In vivo and in the presence of HmT toxin/methomyl, growth on non-fermentable carbon sources is inhibited (Fig. 3) and a significant proportion of respiration-deficient petite colonie mutants accumulates (Table 1). The first phenomenon may simply be the effect of the second one. However, this does not seem probable, since the induction of *rho*$^-$ cells is incomplete (Table I) under conditions (6 mM methomyl) where the arrest of respiratory growth is complete (Fig. 3). It thus seems more plausible to think that the primary effect of the maize protein in yeast mitochondria in the presence of methomyl is the arrest of respiratory growth and that the induction of *rho*$^-$ cells is a consequence of this. It is important to stress that the complete arrest of respiratory growth is not due to the inhibition of cellular respiration, since the oxygen uptake of yeast cells is not inhibited by methomyl, regardless of whether the maize protein is present or not (Fig. 4). The cause of the drastic biological effects must therefore lie elsewhere. The in vitro studies on isolated mitochondria give us a clue to the mode of action of the protein.

The major effect on *urf13-TW* mitochondria is the stimulation of oxygen uptake when NADH is the substrate (Fig. 5). We interpret this as uncoupling since the effect of methomyl mimics that of CCCP, the classic uncoupler. The effect of methomyl alone is almost as strong as that of CCCP itself since subsequent addition of the latter increases the oxygen uptake by 30% to 40% only, as compared with an increase of some 700% in the absence of *urf13-TW* (Fig. 5). Interestingly, when succinate is the substrate there is no stimulation of oxygen uptake (Fig. 5B). Thus the combined action of the maize protein and methomyl singles out the pathway

that originates at NADH from the various pathways of coupling to electron transfer. Yeast contains several types of NADH dehydrogenase activity which may be extra- or intra-mitochondrial and vary in proportion depending on growth conditions (De Vries and Marres 1988; Grivell 1989). We do not know which of the NADH dehydrogenases is involved in the effect of *urf13-TW* but we know that it transfers the electrons via the QH_2:cytochrome *c* oxidoreductase (the bc_1 complex) because the oxygen uptake is completely inhibited by antimycin A both in the presence and the absence of methomyl. This inhibition by antimycin A suggests that the pathway is physiologically important (while the NADH oxidation which is not inhibited by antimycin A may be less important; cf. De Santis and Melandri 1984).

In conclusion, we interpret our observations as follows: the simultaneous presence of the maize mitochondrial 13 kDa protein and HmT toxin/methomyl uncouples the physiologically essential oxidation of NADH; this uncoupling effect completely prevents growth on respiratory substrates like ethanol or glycerol, strongly diminishes growth on glucose and increases the proportion of petite colonie mutants. We believe that this model system will be useful for a better understanding of the phenomenon of maize cytoplasmic male sterility, and be helpful for identifying and isolating nuclear genes in yeast or higher plants that suppress the mitochondrial deficiency in a manner analogous to the restorer genes of maize.

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